AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Directed Molecular Evolution of an Engineered Gammaretroviral Envelope Protein with Dual Receptor Use Shows Stable Maintenance of Both Receptor Specificities

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ABSTRACT

We have previously reported the construction of a murine leukemia virus-based replication-competent gammaretrovirus (SL3-AP) capable of utilizing the human G protein-coupled receptor APJ (hAPJ) as its entry receptor and its natural receptor, the murine Xpr1 receptor, with equal affinities. The apelin receptor has previously been shown to function as a coreceptor for HIV-1, and thus, adaptation of the viral vector to this receptor is of significant interest. Here, we report the molecular evolution of the SL3-AP envelope protein when the virus is cultured in cells harboring either the Xpr1 or the hAPJ receptor. Interestingly, the dual receptor affinity is maintained even after 10 passages in these cells. At the same time, the chimeric viral envelope protein evolves in a distinct pattern in the apelin cassette when passaged on D17 cells expressing hAPJ in three separate molecular evolution studies. This pattern reflects selection for reduced ligand-receptor interaction and is compatible with a model in which SL3-AP has evolved not to activate hAPJ receptor internalization.

IMPORTANCE

Few successful examples of engineered retargeting of a retroviral vector exist. The engineered SL3-AP envelope is capable of utilizing either the murine Xpr1 or the human APJ receptor for entry. In addition, SL3-AP is the first example of an engineered retrovirus retaining its dual tropism after several rounds of passaging on cells expressing only one of its receptors. We demonstrate that the virus evolves toward reduced ligand-receptor affinity, which sheds new light on virus adaptation. We provide indirect evidence that such reduced affinity leads to reduced receptor internalization and propose a novel model in which too rapid receptor internalization may decrease virus entry.

Fusion of lipid membranes is not a spontaneous event and thus requires a supply of energy through an active fusion mechanism. Retroviruses are enveloped viruses and hence are strictly dependent on their fusion machinery for entry into host cells. In retroviruses, including murine leukemia virus (MLV), the fusion energy is stored in the envelope (Env) protein that is present in the membrane envelope surrounding the viral particle. Env consists of trimers of two subunits, the N-terminal surface (SU) subunit and the C-terminal transmembrane (TM) subunit. SU is responsible for recognizing and binding cellular receptor proteins, which differ among virus types and define their tropism. The SU and TM subunits remain associated through noncovalent interactions or, as for MLV, through a disulfide bridge that is broken as a result of disulfide isomerization following receptor binding (1). Here, the fusion energy stored in TM and suppressed by SU is released by conformational changes allowing the formation of an elongated triple helix in TM. This leads to insertion of the fusion peptide into the membrane of the host cell and hence induces membrane fusion (2), as reviewed in references 3 and 4. Other reports have suggested a need for proteolytic cleavage of SU in endosomes to allow viral fusion (5, 6). However, variations have been observed among various cell types showing MLV variants to fuse either directly at the plasma membrane or following internalization through endosomes (5).

Most attempts to retarget retroviral vectors by using modified envelope (Env) proteins have shown that the acquisition of a viral infection close to wild-type levels is not trivial, which indicates that the viral fusion mechanism is very sensitive to alterations (7, 8). Often, retargeting attempts have resulted in chimeric proteins that are capable of binding to the cellular receptors but fail to induce membrane fusion and viral infection to a level close to that of the wild type. An example of this has been reported by Katane et al., who accomplished viral infection through CXCR4 by inserting the ligand human stroma-derived factor 1α (SDF-1α) into Moloney MLV (Mo-MLV) Env but with titers below wild-type levels (9). Among the more successful examples is that of the selection of a retargeted feline leukemia virus (FeLV-A) Env protein by Bupp et al. (10–13) showing that the chimeric Env protein loses its af-

Received 24 August 2015 **Accepted** 19 November 2015

Accepted manuscript posted online 25 November 2015

Citation Friis KP, Iturrioz X, Thomsen J, Alvear-Perez R, Bahrami S, Llorens-Cortes C, Pedersen FS. 2016. Directed molecular evolution of an engineered gammaretroviral envelope protein with dual receptor use shows stable maintenance of both receptor specificities. J Virol 90:1647–1656. doi:10.1128/JVI.02013-15.

Editor: S. R. Ross

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finity for the natural receptor after rounds of genetic selection (11–13).

In addition, Li and colleagues have successfully replaced the receptor-binding domain of Mo-MLV with the ligand of the somatostatin receptor and by this inducing viral infection through the somatostatin receptor with titers equal to that of the wild-type virus (14). However, a later report showed that postbinding restrictions existed, depending on the somatostatin receptor used (15).

We previously constructed the first example of a retargeted replication-competent gammaretrovirus (SL3-AP) capable of infecting through the human G protein-coupled receptor APJ (hAPJ) and the murine Xpr1 receptor with equal efficiencies (16). The replication-competent SL3-AP virus is based on the SL3-2 Env protein, which uses the xenotropic-polytropic receptor (Xpr1) for entry but, unlike other similar viruses, has a tropism restricted to rodent cells. SL3-AP was shown to be capable of replicating in cells expressing either of its two receptors, to cause resistance to superinfection, and also to induce complete downregulation of hAPJ in infected cells.

hAPJ is expressed in various tissues, such as those of the brain, kidney, liver, lung, gastrointestinal tract, and cardiovascular system, as reviewed in references 17 and 18. The receptor is implicated in several diseases, such as cardiovascular diseases, obesity, and diabetes (18), and is also known to function as a coreceptor for HIV-1 (19–21), which makes hAPJ a suitable candidate for retargeting studies.

Significant for the apelin receptor is its relationship with its natural ligand, apelin. Apelin is found in different molecular forms; among these are endogenous apelin-36, apelin-17, and apelin-13, which are 36, 17, and 13 amino acids long, respectively, as well as a pyroglutamyl form of apelin-13 named pE13F. These ligands interact differently with APJ regarding binding affinity, subsequent internalization, and recycling of the receptor (21–23). For this study, apelin-13 which is conserved between humans and rats, was selected because of its documented high activity when interacting with the apelin receptor, as well as its small size. Apelin-13 has a high affinity for hAPJ (10^{-10} M), but the ligand is less potent than apelin-17 at APJ binding (10^{-11} M), inhibition of cyclic AMP (cAMP) production, and activation of APJ internalization (21, 24, 25). The binding of apelin-13 results in receptor internalization through a clathrin-mediated pathway, followed by recycling back to the cell surface (26). The longer apelin-36 peptide, on the other hand, triggers receptor internalization and subsequent degradation (22).

Considering APJ internalization, several studies have shown that the C-terminal phenylalanine (F13 in apelin-13) is extremely important for the internalization of the receptor (23, 27). This phenylalanine has previously been shown by Iturrioz et al. to be embedded in a hydrophobic pocket at the bottom of the APJ binding site (23).

The SL3-2 Env protein was modified to include the smaller apelin-13 peptide in one of the variable regions (VRB) of the receptor binding domain, surrounded by two flexible linker sequences (Fig. 1A) previously shown to be necessary for the correct presentation of apelin-13 in Mo-MLV Env (28).

The possible coevolution of the viral Env protein and its relevant host cell entry receptor might be influenced by the internalization pathway of the cellular receptor. Several studies have documented the importance of the internalization through specific

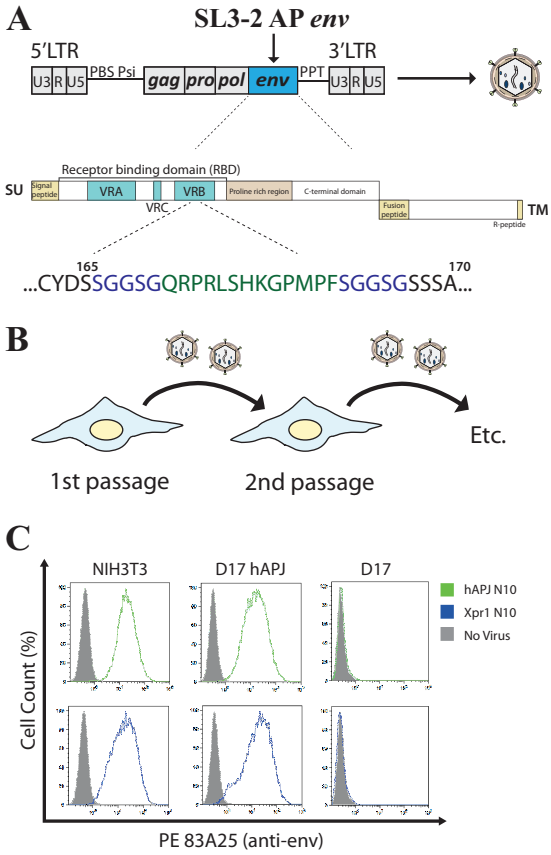

FIG 1 Molecular evolution of SL3-AP. (A) Schematic showing the location and amino acid sequence of the apelin cassette in the SL3-2 Env protein (16). VRA, VRB, and VRC are variable regions A, B, and C, respectively. The apelin sequence is green, and the linker sequence is blue; the black sequences are of SL3-2 origin. LTR, long terminal repeat. (B) Illustration of the strategy used for molecular evolution studies with SL3-AP. (C) Infectivity after passaging of SL3-AP on cells expressing one of the two receptors, i.e., Xpr1 on NIH 3T3 cells or hAPJ on D17 hAPJ cells. The infectivity of the passaged virus Xpr1 N10 and hAPJ N10 was measured on NIH 3T3, D17 hAPJ, and D17 cells by flow cytometry with an antibody against the MLV Env protein. N10 indicates that the virus has been through 10 passages in the respective cell line. The data are representative of three independent experiments. PE, phycoerythrin.

cellular compartments in relation to viral infectivity (5, 15). Passaging of virus on permissive cells specific for a function or feature of interest can result in the fixation of particular mutations. Zavorotinskaya and Albritton passaged a replication-competent MLV on cells expressing receptors with a binding deficiency, isolated the *env* genes from representative quasispecies capable of infecting and surviving in these cells, and analyzed the *env* genes for mutations (29). This resulted in the identification of key residues in Env that are essential for receptor interactions. Another example is that of Ferrarone et al., who passaged an ecotropic Mo-MLV variant on restrictive cells, which resulted in the identification of residues important for receptor affinity (30).

Realizing that the SL3-AP virus might have potential for better adaptation to APJ as an entry receptor, we have monitored the molecular evolution of the chimeric SL3-2 AP Env protein over several passages and under selection to infect through APJ. We found that the chimeric Env protein gene exhibited mutations in the apelin cassette but that the virus did not lose its dual tropism or

evolve to utilize other entry receptors on canine cells, even after more than 10 passages.

Interestingly, the results are compatible with the model in which SL3-AP has evolved not to activate hAPJ receptor internalization.

MATERIALS AND METHODS

Apelin peptides. Apelin peptides were synthesized by Schafer-N (Copenhagen, Denmark), and the samples obtained were >95% pure.

The longer peptides (SL3-2 AP [Ac-CYDSSGGSGQRPRLSHKGPMP FSGGSGSSSAQGATPGGRC-OH] and SL3-2 AP F183L [Ac-CYDSSGGS GQRPRLSHKGPMPLSGGSGSSSAQGATPGGRC-OH]) were cyclized with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] according to previously published guidelines (31). Briefly, the peptides were dissolved in a solution of 50 mM sodium acetate, 2 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and 1 M Tris (pH 8.0) to a concentration of 0.001 M.

The reaction was allowed to proceed for 30 min at room temperature, and the cyclized peptides were separated by high-performance liquid chromatography and analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Construction of the EGFP-tagged human apelin receptor. The full-length coding sequence of the human apelin receptor was isolated by PCR from a human APJ-expressing plasmid (pCDNA-APJ, a kind gift from R. J. Pomerantz) and inserted between the EcoRI and AgeI restriction sites of pEGFP-N1 (Clontech, Palo Alto, CA) upstream of the 5′ end of the enhanced green fluorescent protein (EGFP) cDNA as previously described by El Messari et al. and De Mota et al. (26, 32).

D17 hAPJ-EGFP cells were made by transfection of the plasmid and selection with G418 at 600 μg/ml until a resistant culture emerged. The selected population was sorted for high expression of hAPJ-EGFP by fluorescence-activated cell sorting.

Cell cultures. CHO-K1 cells (American Type Culture Collection, Manassas, VA) stably expressing rAPJ-GFP (rat APJ coupled to EGFP) (32) were grown in Ham's F12 medium with 10% (vol/vol) fetal calf serum. D17 cells and D17 cells stably expressing hAPJ (28) or hAPJ-EGFP were grown in minimum essential medium alpha with GlutaMAX-1 and 10% (vol/vol) fetal calf serum. NIH 3T3 cells and the semipackaging cell line CeB (33) were grown in Dulbecco's modified Eagle's medium with GlutaMAX-1 and 10% (vol/vol) newborn calf serum.

All growth media contained 100 U of penicillin/ml and 100 μg of streptomycin/ml. All cells were incubated at 37°C in 90% relative humidity and 5.0% CO_2.

Envelope design. Briefly, the mutant envelope construct SL3-2 AP F183L was cloned into a bicistronic vector (33) expressing the Env protein, the neomycin phosphotransferase II (Neo) selection marker, and an internal ribosome entry site by standard cloning techniques. The amino acid sequence inserted into SL3-2 AP Env at position 165 (16) is SGGSG QRPRLSHKGPMPLSGGSGSS (the target sequence is underlined).

The resulting vector was cotransfected into HEK293T cells with a *gag-pol* expression plasmid to result in infectious virions. The virions were used to transduce the CeB semipackaging cell line (33). A stably expressing cell line emerged when cells were selected in medium containing G418 at 600 μg/ml.

Pseudotyping. The virions were pseudotyped with vesicular stomatitis virus glycoprotein G (VSV-G) and the chimeric SL3-2 Env proteins by cotransfecting HEK293T cells with a VSV-G expression plasmid and one of the chimeric SL3-2 Env-expressing vectors along with a *gag-pol* expression plasmid to result in infectious virions. These were used to infect D17 cells expressing hAPJ. After infection, the cells were selected in medium containing G418 at 600 μg/ml for approximately 10 days. The amount of Env expressed on the surface of the resulting cells was measured by flow cytometry analysis with an anti-Env antibody.

Infectivity assays. The supernatant of infected CeB cells was sterile filtered and added to the target cells in serial 10-fold dilutions with Polybrene at 6 μg/ml. At 24 h postransduction, the cells were subjected to selection in medium containing G418 at 600 μg/ml until colonies emerged. The titer in CFU was calculated by counting the colonies that emerged. All titer measurements were reproduced in at least three independent experiments.

Molecular evolution assay. Viral passages were created by transferring undiluted and sterile filtered supernatant from a confluent T80 flask with infected cells and added Polybrene at 6 μg/ml to uninfected NIH 3T3 or D17 APJ cells. The cells were grown for 2 to 4 weeks, and infection was verified by flow cytometry analysis with an anti-MLV Env antibody.

Genomic DNA was isolated from infected cells by DNAzol Reagent (Invitrogen) according to the manufacturer's instructions. SL3-2 *env* was amplified by PCR. The PCR products were sequenced with SL3-2 *env*-specific primers.

Flow cytometry analysis. Cells were harvested with 0.05% trypsin-EDTA solution (Gibco). A total of 10^6 cells were washed with phosphate-buffered saline (PBS) containing 2% newborn calf serum and incubated for 45 min at 4°C with 200 μl of supernatant from 83A25 hybridoma cells for envelope labeling (34). The cells were washed twice in PBS containing 2% newborn calf serum and incubated with 5 μl of 1:40-diluted secondary antibody (goat anti-rat IgG R-phycoerythrin conjugate; Southern Biotechnology Associates, Inc.) for envelope labeling for 45 min at 4°C. The cells were washed in PBS containing 2% newborn calf serum and resuspended in 750 μl of 2% paraformaldehyde dissolved in PBS at pH 7.4. The samples were analyzed on a Beckman Coulter Gallios Flow Cytometer.

Membrane preparation. Membrane preparation from stably transfected D17 hAPJ cells was carried out as previously described (35). Cells were grown in tissue culture dishes (245 by 245 by 25 mm) until they were 90% confluent. All subsequent steps were performed on ice. The cells were washed with 50 ml of ice-cold PBS and harvested by scraping into 60 ml of ice-cold PBS with 1 mM EDTA. The cells were collected by centrifugation at 400 × *g* for 10 min. The cells were homogenized in 6 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 10% (wt/vol) sucrose, 6.5 mM $MgCl_2$ with Complete EDTA-free protease inhibitor cocktail (Roche), and 0.3 mg/ml Bacitracin (Sigma-Aldrich) with a Teflon-glass homogenizer (five strokes at 2,000 rpm) and then centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was kept on ice, and the corresponding pellet was subjected to a second identical homogenization step and centrifugation. Both supernatants were pooled and centrifuged again at 100,000 × *g* for 100 min. The crude membrane pellet was washed in 3 ml of buffer to eliminate any soluble material. The sample was resuspended in 1 ml of 50 mM HEPES buffer, pH 7.5, containing 5 mM $MgCl_2$ and homogenized by sonication for 10 s, and the membrane preparations were kept at −80°C until use.

The protein concentration of the membrane preparations was estimated with the Pierce BCA protein assay kit (Thermo Scientific) in accordance with the guidelines of the manufacturer.

Radioligand binding assay. The membrane preparation (approximately 1 μg of total mass of membrane proteins/sample) was incubated with 2×10^{-10} M ^{125}I-pE13F (AP13) ligand (PerkinElmer) in binding buffer (50 mM HEPES, 5 mM $MgCl_2$, 1% [wt/vol] bovine serum albumin [BSA], pH 7.4 to 7.5) alone or in the presence of increasing concentrations of different apelin peptides at 20°C. The reaction was stopped after 45 to 60 min with a large excess of ice-cold binding buffer, and the samples were filtered on Whatman GF/C filters. After the filters were washed, radioactivity was counted on a Wallac Wizard 1470 Gamma Counter (PerkinElmer).

Saturation binding curves were obtained by incubating membrane preparations (1 μg of total mass of membrane proteins/assay) for 60 min at 20°C with the radioligand ^{125}I-pE13F at different concentrations (10^{-10} to 10^{-8} M) in binding buffer alone or in the presence of 10^{-6} M K17F. The data were analyzed by the GraphPad Prism software program.

cAMP assay. D17 cells stably expressing hAPJ were suspended in stimulation buffer (Hanks balanced salt solution [Life Technologies], 5 mM HEPES [Life Technologies], 0.1% BSA [PerkinElmer], 1 mM 3-isobutyl-1-methylxanthine [Sigma-Aldrich]) and dispensed into a 384-well plate

with approximately 1,000 D17 hAPJ cells per well (5 μl/well). A 5-μl volume of stimulation buffer containing 10 μM forskolin and apelin peptides at various concentrations was added to the cells, and the mixture was incubated for 20 min at 37°C. cAMP levels were quantified with the cAMP dynamic 2 assay kit (Cisbio Bioassays, Codolet, France) based on homogeneous time-resolved fluorescence (HTRF) technology. The HTRF signal was counted with an EnVision Xcite microplate reader (PerkinElmer).

Internalization assay. The internalization assay was performed as previously described (26). Briefly, CHO rAPJ-EGFP (rat APJ coupled to EGFP) or D17 hAPJ-EGFP cells were used to seed 0.01% (wt/vol) polylysine (Sigma-Aldrich)-coated 16-well Lab-Tek chamber slides (Nunc) at approximately 2.5×10^4/well. Cells were incubated for 30 min at 37°C in 90% relative humidity and 5.0% CO_2. The cells were incubated with 90 μM cycloheximide (Sigma-Aldrich) at 37°C for 1.5 h. Cycloheximide was maintained in the medium during the subsequent steps to ensure continued prevention of *de novo* protein synthesis. The cells were then washed in ice-cold Earle's buffer (0.14 M NaCl, 5.1 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 24.9 mM HEPES) supplemented with 0.2% BSA, 0.1% D-glucose, and 90 μM cycloheximide. Cells were then incubated with increasing concentrations of apelin peptides diluted in Earle's buffer for 20 min on ice. Internalization was then triggered by transferring the cells at 37°C for 20 min. The cells were washed in ice-cold PBS and fixed with 4% paraformaldehyde dissolved in PBS for 10 min at 20°C. The wells were then washed in ice-cold PBS, mounted in Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA, USA), and covered with a coverslip for confocal microscopy analysis.

Confocal microscopy. Cells were examined with a Leica TCS SP5 MP (Leica Microsystems, Heidelberg, Germany) equipped with an argon ion laser adjusted to 488 nm for GFP and the spectrophotometer set to acquire emission between 530 and 540 nm. Sections were scanned with a photomultiplier and the appropriate filter for detection of fluorescence from GFP. Optical sections (1,024 by 1,024 pixels) of individual cells were taken at the equatorial level (level of the nucleus) with a 63× oil immersion objective with acquisition software (LAS AF; Leica).

RESULTS

Continued passaging of SL3-AP does not influence the dual tropism of the virus. To further study determinants of its novel receptor usage and to improve retargeting efficiency, we subjected the replication-competent SL3-AP virus to molecular evolution studies through subsequent passages on cells expressing one or the other of its two receptors. The absence of one of its two receptors may lead to a loss of functional interaction with the missing receptor, since this function is no longer beneficial for the virus.

Since the SL3-AP virus was previously shown to replicate in canine D17 cells in an hAPJ-dependent manner (16), D17 cells stably expressing hAPJ were used for the molecular evolution study together with the murine cell line NIH 3T3, which contains the natural polytropic-xenotropic Xpr1 receptor for SL3-2. To study the adaptation of the apelin cassette when it is exposed to repeated passaging through one of its two receptors, both cell types were transduced with sterile filtered viral supernatant from transduced NIH 3T3 cells (16). In each passage, viral infection was allowed to establish itself for approximately 2 to 4 weeks after infection from the previous passage as illustrated in Fig. 1B. The presence of viral infection was verified by flow cytometry analysis with an antibody against the MLV Env protein. When infection was verified, the supernatant was used to passage the virus to uninfected cells.

Passages in murine NIH 3T3 cells expressing native Xpr1 or in canine D17 cells expressing hAPJ did not influence the infectivity through Xpr1 or hAPJ, as shown in Fig. 1C. After 10 passages in cells expressing one of the receptors, the virus was still able to infect through the other, indicating that the virus has not adapted to its current environment by losing its ability to infect through the alternate receptor. At the same time, after 10 passages in D17 cells expressing hAPJ, the SL3-AP virus remained unable to infect D17 cells that lacked both hAPJ and murine Xpr1, suggesting that utilization of a new receptor molecule has not evolved.

Passaging of SL3-AP under selection pressure to infect through hAPJ results in mutations in SL3-2 AP Env. The viral enzyme reverse transcriptase frequently introduces nucleotide changes into the genome, which can be fixed in the population upon encountering appropriate selection pressures, when a retrovirus is monitored through several passages (29). Passaging of SL3-2 AP on D17 hAPJ cells allowed us to monitor the genetic evolution of the virus in order to identify residues in the viral Env protein that are important for receptor binding and viral infection. The *env* genes of the passaged virus populations were isolated from genomic DNA from infected D17 APJ cells and analyzed for emerging mutations by PCR, followed by DNA sequencing, both with primers specific for MLV *env*.

Three separate molecular evolution studies (studies I to III) of SL3-AP on D17 cells expressing hAPJ have revealed a pattern of recurrent mutations, as shown in Fig. 2.

Interestingly, one of the mutations occurs in the apelin cassette, resulting in a transition from a thymine to a cytosine. This leads to a substitution where the last phenylalanine of the apelin-13 sequence is changed into a leucine, F183L (the number refers to the residue position in the SL3-2 AP Env protein).

The other mutation occurs outside the apelin cassette. The transition from a guanine to an adenine results in a silent mutation V440V. Since the amino acid does not change, it is less likely for this mutation to have a large impact on infectivity. However, this valine is conserved in related MLVs and the transition from a guanine to an adenine resembles the codon use in xenotropic MLV (X-MLV), as well as in amphotropic MLV, as revealed by alignment of sequences of the MLV variants (data not shown). Although its selection in three independent passages implies a function, the impact of the V440V mutation is not assumed to be related specifically to the function of the Env protein or the F183L substitution and was therefore not studied further.

The F183L and V440V mutations evolve in response to the persistent selection of infection through APJ since they are not seen when virus passaged in NIH 3T3 cells is transferred to D17 hAPJ cells for a single round of infection before sequence analysis. Hence, these mutations are due to the specific selection in D17 hAPJ cells.

The F183L mutation in SL3-2 AP does not detectably influence the infectivity or surface expression of the chimeric Env protein. The F183L substitution that arose in the apelin cassette during passaging in D17 hAPJ cells is noteworthy because of the specific position of the mutation in the apelin-13 ligand. Interestingly, substitution or deletion of the C-terminal phenylalanine of apelin-13 has been shown to decrease binding or activity (36, 37) while the same deletion or substitution in K17F only decreases the ability of the peptide to trigger receptor internalization without affecting binding (23). Thus, F183L could be expected to affect the infectivity of the SL3-2 AP Env protein.

A possible way to investigate significant changes in infectivity induced by the F183L mutation is to clone a recombinant SL3-2 AP Env protein that expresses the F183L mutation and measure the infectivity of the chimeric Env protein by titer determination

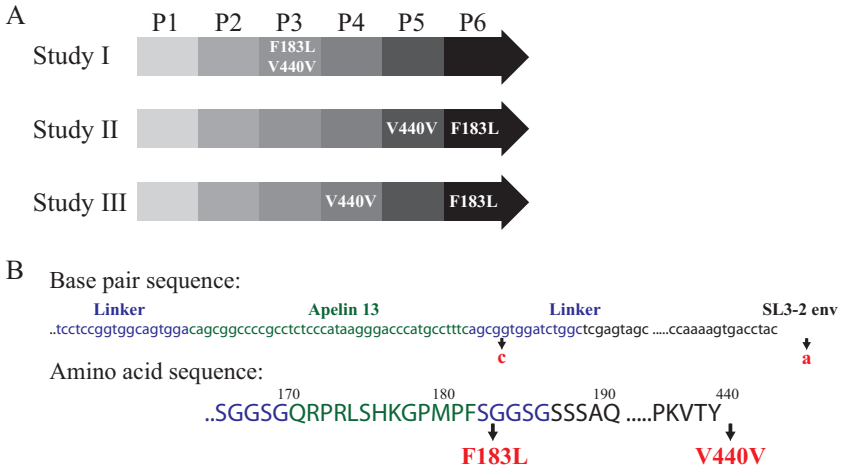

FIG 2 Selected amino acid changes found in hAPJ molecular evolution studies. (A) Alignment of genomic DNA sequences from 10 passages of SL3-AP in D17 APJ cells reveal recurrent point mutations. The selected mutations are shown on the arrows in the passages (P1 to P6) where they became prevalent and not in the passages where they first arose. (B) The positions of the mutations in the SL3-2 AP Env protein are illustrated on the base pair level, as well as in the amino acid sequence. Black, SL3-2 Env; blue, SGGSG linker sequence; green, apelin-13 ligand. The names refer to the positions in the amino acid sequence of SL3-2 AP Env, as illustrated.

as described elsewhere (33). Briefly, titer measurements were conducted with a minivirus system consisting of a bicistronic vector expressing the chimeric Env protein, which is cotransfected with a *gag-pol* expression plasmid into 293T cells (33, 38). The resulting virions were used to transduce an NIH 3T3-derived semipackaging cell line, CeB, stably expressing *gag-pol*. The viral supernatant from the transduced and selected cells was added to D17 hAPJ and NIH 3T3 cells.

The results in Fig. 3 show that the potential variations in infectivity between the mutant Env protein and the original SL3-2 AP Env protein or the wild-type SL3-2 Env protein were not detectable in this assay. This indicates that if the infectivity of the chimeric Env proteins was affected by the mutations, the effect is too small to be measured by standard titer assays.

The F183L substitution could also influence the assembly or release of the newly produced viral particles from the cell. As a measure of this, we used flow cytometry analysis with an antibody against the viral Env protein to investigate the surface expression of Env on cells infected with virions expressing the chimeric Env proteins and pseudotyped with VSV-G. The results showed small variations in labeling; however, no significant difference in Env surface expression was seen among cells infected with wild-type SL3-2, SL3-2 AP, or mutant SL3-2 AP (Fig. 4). This indicates that the F183L substitution does not result in cytoplasmic retention of the viral Env protein.

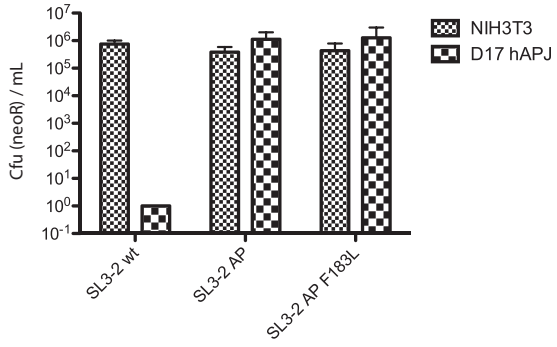

FIG 3 Infectivity of the SL3-2 AP mutants expressed in the minivirus system on noninfected NIH 3T3 or D17 hAPJ cells measured as neomycin resistance (neoR). No significant variation is registered on NIH 3T3 or D17 hAPJ cells. wt, wild type.

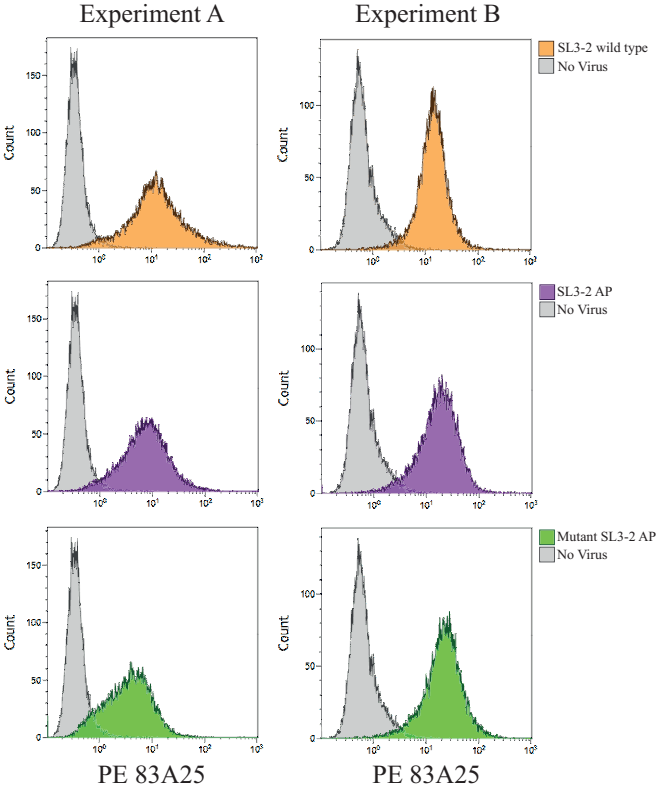

FIG 4 Surface expression of Env on D17 hAPJ cells infected with virions expressing the chimeric Env proteins and pseudotyped with VSV-G. Data from two independent experiments out of four are shown.

TABLE 1 Peptide binding affinities and cAMP production determined with human apelin receptor D17 cells

Peptide	Amino acid sequence	Mean binding affinity (K_i, nM) ± SEM[a]	Mean cAMP production (IC_{50}, nM) ± SEM[b]
K17F	H-KFRRQRPRLSHKGPMPF-OH	1.25 ± 0.8[c]	2.03 ± 0.59[d]
pE13F[e]	H-pERPRLSHKGPMPF-OH	2.49 ± 2[c]	0.46 ± 0.11[c]
G14F	H-GQRPRLSHKGPMPF-OH	0.06 ± 0.01	0.34 ± 0.07
G14L	H-GQRPRLSHKGPMPL-OH	3.29 ± 1[d]	4.35 ± 2.39[c]

[a] Four independent experiments were performed in duplicate.
[b] Three independent experiments were performed in duplicate.
[c] Not significantly different from G14F treatment.
[d] Significantly different from G14F treatment ($P < 0.05$).
[e] pE13F is the pyroglutamyl form of apelin-13.

Binding affinity of G14F for hAPJ is decreased by the F183L substitution. The nature of the apelin ligand, regarding the number and type of amino acids, has previously been reported to be crucial for the activity of the peptide in relation to APJ (21–23). Hence, the effect of the F183L substitution in G14F on its ability to bind to hAPJ was investigated. We first determined the binding capacities of the 14-amino-acid apelin ligands mimicking the cassette in SL3-AP with (G14L) or without (G14F) the F183L mutation. The binding capacities were measured together with those of apelin-17 (K17F) and apelin-13 (pE13F), two endogenous molecular forms of apelin known to have nanomolar affinities for the apelin receptor (Table 1). The peptides investigated are named according to the first amino acid, the length of the peptide, and the last amino acid.

We determined the apparent dissociation constant (K_d) and the maximal receptor density (B_{max}) by determining saturation curves with D17 hAPJ membrane preparations and increasing concentrations of ^{125}I-pE13F as a radioligand (from 10^{-10} to 2.3×10^{-9} M). The K_d and B_{max} values for ^{125}I-pE13F were 0.20 nM and 3 pmol/mg (Fig. 5), which are comparable to previously reported K_d and B_{max} values for CHO hAPJ membrane preparations (K_d, 1 nM; B_{max}, 7.76 pmol/mg) (23).

Then we characterized the binding affinities of mutated apelin peptides for hAPJ by incubating D17 hAPJ membrane preparations with 0.2 nM ^{125}I-pE13F and various concentrations of the peptides (10^{-6} to 10^{-12} M). Under our experimental conditions, the addition of a glycine residue to the N-terminal part of pE13F (G14F) improves its affinity by a factor of 41 over that of pE13F.

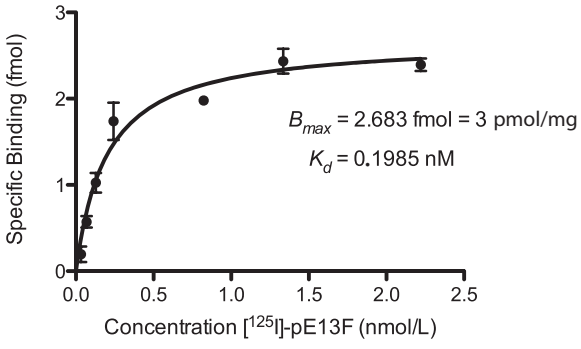

FIG 5 ^{125}I-pE13F saturation binding curve for a D17 hAPJ membrane preparation. The saturation experiment was conducted with D17 hAPJ membrane preparations and increasing concentrations of ^{125}I-pE13F with or without 10^{-6} M K17F to define specific binding. K_d and B_{max} were deduced from the specific binding curve.

The F183L substitution in G14F (G14L) (Table 1) induced a significant 55-fold decrease in the binding affinity of G14L below that of G14F.

Inhibition of forskolin-induced cAMP production by G14F is decreased by the F183L substitution. To further characterize the effect of the F183L substitution, we evaluated the ability of the peptides to inhibit forskolin-induced cAMP production in D17 cells stably expressing hAPJ. The ability of the peptides to inhibit forskolin-induced cAMP production reflects their ability to activate apelin receptor G_i signaling.

The results are shown in Table 1. Interestingly, the most active peptide in relation to the D17 hAPJ cell line was again G14F, which is the original apelin insertion into SL3-2 AP. The least active peptide was the G14L, which is the product with the F183L substitution seen in all of the studies of molecular evolution in D17 hAPJ cells. However, the effect of the F183L substitution on the inhibition of forskolin-induced cAMP production was not as marked as in the binding studies, since the activity was only 10 times as low as that of G14F.

Thus, the change in binding affinity resulting from the mutations might be the main reason for the selectivity of especially F183L.

The F183L substitution reduces the ability of G14F to trigger APJ-GFP internalization. Under normal conditions, stimulation of the APJ receptor by K17F or pE13F induces internalization of the receptor. Depending on the nature of the ligand, the receptor either recycles to the cell membrane or is targeted for degradation (28, 39). The time it takes for the G protein-coupled receptor to recycle or become degraded is a factor that could influence the receptor interaction of the virus. Hence, we decided to investigate the effect of the F183L substitution on the capacity of the peptide to trigger apelin receptor internalization.

Apelin receptor internalization was monitored by confocal microscopy analysis of CHO cells expressing rAPJ tagged at its C terminus with EGFP. CHO rAPJ-EGFP cells have previously been successfully used to measure APJ internalization (23, 25, 26, 32). Our results are shown in Fig. 6.

In the absence of ligand, intense rAPJ-EGFP fluorescence is seen at the plasma membrane of the cells, showing the presence of the receptor at the cell surface. Treatment with pE13F at a concentration of 10^{-6} M results in a marked decrease in fluorescence at the plasma membrane and the appearance of many fluorescent intracytoplasmic vesicles. The endogenous peptide pE13F at 10^{-6} M, similar to a concentration of 10^{-7} M, as previously shown (25, 26), is a potent inducer of rAPJ-EGFP internalization (Fig. 6). Similarly, G14F induced strong inter-

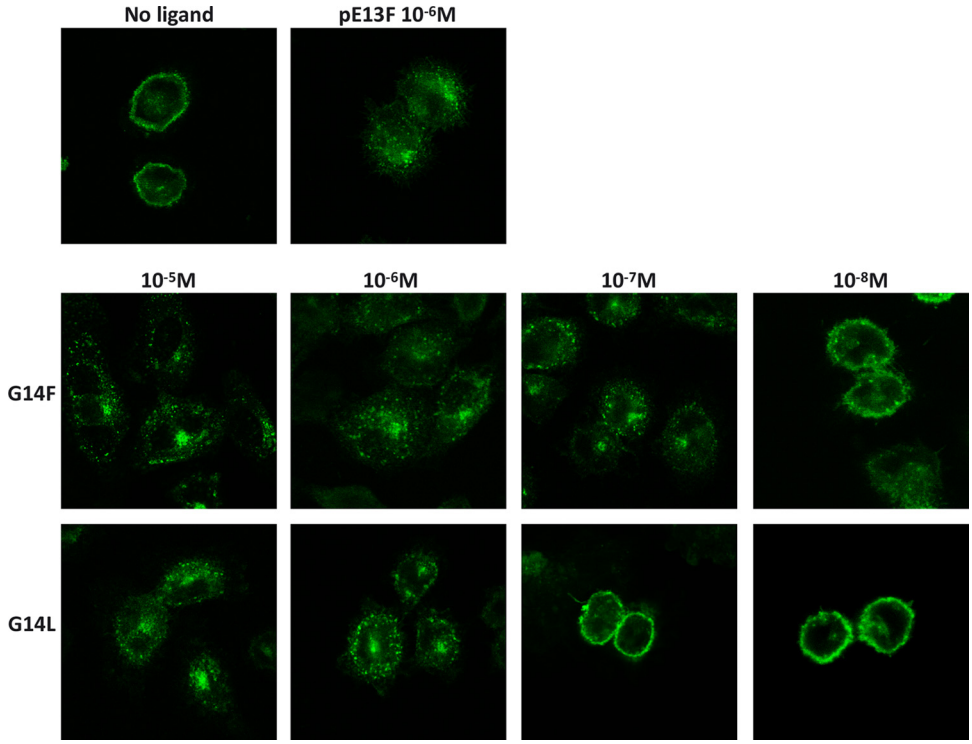

FIG 6 Internalization of rat apelin receptor-EGFP by apelin peptides G14F and G14L. CHO cells stably expressing rat apelin receptor-EGFP were stimulated with different concentrations of apelin peptides G14F and G14L as indicated. The cells were fixed and analyzed by confocal microscopy. The results are representative of several independent experiments.

nalization of rAPJ-EGFP at concentrations of 10^{-7} to 10^{-5} M. In contrast, G14L induced no internalization of rAPJ-EGFP at concentrations of 10^{-8} to 10^{-7} M (Fig. 6). The effect on APJ internalization was measured on D17 cells expressing hAPJ tagged at its C terminus with fluorescent EGFP to relate the experiments to the results obtained with the SL3-2 AP Env protein (Fig. 7). Similar to the results obtained with CHO rAPJ-EGFP cells, G14F (10^{-8} to 10^{-5} M) applied to D17 cells

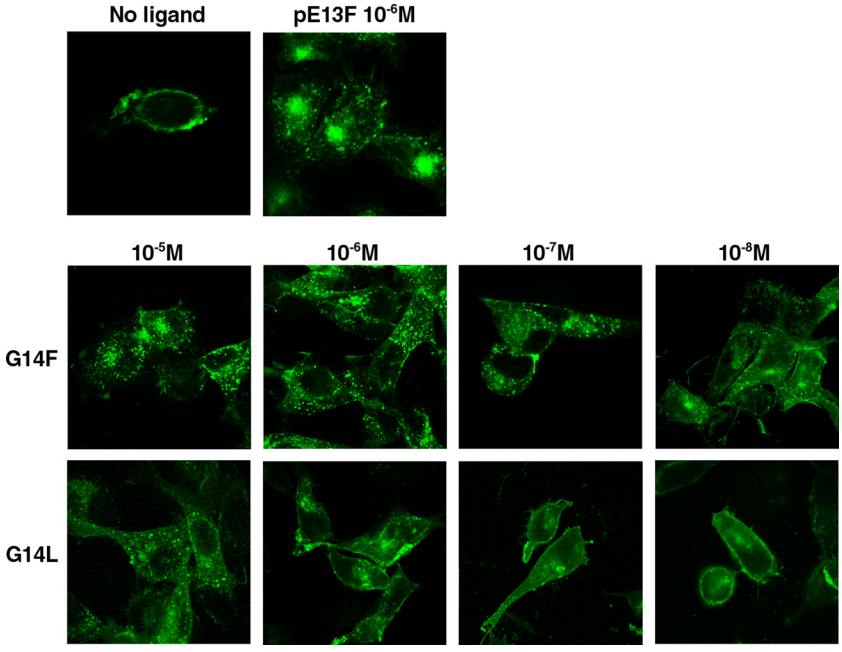

FIG 7 Internalization of human apelin receptor-EGFP by apelin peptides G14F and G14L. D17 cells stably expressing human apelin receptor-EGFP were stimulated with different concentrations of apelin peptides G14F and G14L as indicated. The cells were fixed and analyzed by confocal microscopy. The results are representative of five independent experiments.

TABLE 2 Cyclized peptide binding affinities and cAMP production determined with human apelin receptor D17 cells

Peptide	Amino acid sequence	Mean binding affinity (K_i, μM) ± SEM[a]	Mean cAMP production (IC_{50}, μM) ± SEM[b]
SL3-2 AP	CYDSSGGSGQRPRLSHKGPMPFSGGSGSSSAQGATPGGRC	5.2 ± 1.8	5.8 ± 1.8
SL3-2 AP F183L	CYDSSGGSGQRPRLSHKGPMPLSGGSGSSSAQGATPGGRC	85 ± 39[c]	97 ± 39[c]

[a] Four independent experiments were performed in duplicate.
[b] Three independent experiments were performed in duplicate.
[c] Significantly different from G14F treatment ($P < 0.05$).

stably expressing hAPJ-EGFP induced strong hAPJ-EGFP internalization starting at a concentration of 10^{-8} M, whereas G14L was only capable of inducing clear hAPJ-EGFP internalization at a concentration of 10^{-5} M.

Thus, these data indicate that the F183L substitution strongly influences the internalization of the apelin receptor, in agreement with the decrease in the binding affinity of the G14L peptide for the apelin receptor.

Cyclization of the apelin peptides reduces the interactions with the apelin receptor. The interactions between the apelin cassette in SL3-2 AP and the apelin receptor might be different from that of the linear apelin peptides and the apelin receptor because of the structural constraints of the ligand in the context of the Env protein. We therefore decided to mimic the protein loop in SL3-2 AP in which the apelin peptide was introduced. For this purpose, we designed cyclized peptides with the G14F and G14L peptides flanked by the sequence of the Env loop and connected through a disulfide bridge.

The cyclized peptides were tested for binding affinity and inhibition of forskolin-induced cAMP production (Table 2). The cyclization and the addition of the linkers on the G14F and G14L sequences strongly decreased the K_i of the peptides compared to those of linear G14F and G14L by factors of 87,000 and 26,000, respectively.

In addition, the F183L substitution resulted in a 10-fold reduction in both the K_i and the 50% inhibitory concentration (IC_{50}) in comparison with those of G14F, which is comparable to the results obtained with the linear peptides.

DISCUSSION

We have previously constructed a gammaretrovirus Env protein capable of infecting through either the human G protein-coupled receptor hAPJ or the murine G protein-coupled receptor Xpr1 (16). Here, we have monitored the adaptation of the Env protein when the virus is passaged through one of its two receptors. We were especially focused on the adaptation of the apelin cassette when the virus was passaged on canine D17 cells expressing hAPJ to investigate the importance of the specific residues in relation to interactions with the cellular receptor. The adaptation to this specific functional selection was monitored through more than 10 passages and in three separate studies. A pattern of recurrent mutations was observed in all three studies.

The V440V mutation was seen in SL3-2 Env outside the apelin cassette in all three studies. The codon change of the silent V440V mutation is not expected to induce any specific changes in viral infectivity for D17 hAPJ cells, since the transition from a guanine to an adenine is comparable to the codon use of xenotropic and amphotropic MLVs.

One of the mutations arose in the sequence encoding the apelin ligand, leading to the F183L substitution at the last amino acid of the apelin-13 sequence. Interestingly, this particular phenylalanine has previously been reported to be important for the ability of apelin-13 and K17F to trigger APJ internalization (23, 26).

The same change was seen in the base pair sequence of F183L in all four studies and results in a transition from a thymine to a cytosine; TTC becomes CTC. This could indicate that the mutation might already be present in a subpopulation of the primary virus stock used to start the four passages. However, to make the F183L substitution by a single point mutation, the transition from T to C is the only option, since the other leucine-encoding codons, TTA, TTG, CTT, CTA, and CTG, would require at least two nucleotide substitutions from TTC, which are less likely to occur. At the same time, the F183L substitution is not seen when virus populations passaged on NIH 3T3 cells are transferred to D17 hAPJ cells for a single round of infection. This indicates that the mutation arose in response to infection through hAPJ and not because of the passaging of the virus in general. Hence, the F183L substitution is expected to provide a selective advantage for the mutated SL3-2 AP virus populations in relation to infectivity through specific interactions with the apelin receptor. Miyakawa et al. have previously shown that the specific sequence and hence folding of a ligand (SDF-1α) incorporated into the Mo-MLV Env protein affect the infectivity of the viral vector (40); the F183L substitution might induce equivalent structural changes in SL3-2 AP Env.

To establish whether the F183L mutation results in significant changes in infectivity, a conventional titer assay was used. The infectivity of the Env protein with or without the F183L substitution was measured on both NIH 3T3 and D17 cells expressing hAPJ. However, the conventional titer assay was not sensitive enough to measure a potential effect of F183L on infectivity.

Another potential effect of the F183L mutation could be on the assembly or exit of the virions after infection. The possibility of an assembly deficiency created by the F183L substitution was eliminated by pseudotyping the virions with VSV-G and monitoring the level of surface-expressed Env on infected cells. Pseudotyping with VSV-G eliminates potential variations in viral infection. After a few rounds of infection, small variations in labeling were observed; however, the data did not indicate differences in the assembly process.

Since conventional assays were not able to reveal any changes in infectivity or assembly of the mutant, other assays were introduced into this study. Instead, the effect of the F183L mutation was measured in a series of APJ activity assays since variations in receptor interactions and especially internalization previously have been shown to influence viral infectivity (5, 15). These APJ activity assays showed that the F183L mutation results in decreased APJ affinity, as well as a decreased ability to activate APJ upon binding. It was further investigated whether this resulted in changes in the internalization of APJ. This was the case, since the

F183L substitution decreases the ability of the peptide to induce APJ internalization.

If the effects observed at the peptide level were transferable to virions, the F183L substitution might result in weaker interactions between SL3-2 AP and APJ. We propose that the F183L substitution is responsible for changing the balance between nonproductive internalization leading to APJ-targeted degradation in the lysosome and productive infection resulting from viral membrane fusion at the plasma membrane or the endosomal membrane.

Previous studies have shown that the receptor choice and especially the internalization pathway of the cellular receptor might influence the infectivity and intracellular fate of the virus (15). Hence, we hypothesize that SL3-AP is internalized along with the apelin receptor instead of following the normal internalization/fusion pathway for SL3-2.

Another explanation could relate to the differences in the fate of the apelin receptor after internalization because of variations in the molecular form of the apelin ligands. As mentioned in the introduction, interactions with apelin-13 result in recycling of the receptor, whereas binding of apelin-36 results in degradation of the receptor (22, 26). These differences in the fate of the receptor might influence viral infectivity since studies have shown the internalization of virus through specific cellular compartments to be important for infectivity (5, 15). Krueger and Albritton have previously shown that a chimeric MLV Env protein expressing the somatostatin ligand changes infectivity according to the somatostatin receptor variant used for internalization because of variations in receptor internalization through different cellular compartments (15). Hence, the differences observed in the mutant SL3-2 AP might influence the internalization of the apelin receptor and thus the infectivity of the virus.

Altogether, SL3-AP stably maintains its dual receptor affinity after selection pressure to use only one of the two. Moreover, the results are compatible with the model in which SL3-AP has evolved not to activate hAPJ receptor internalization.

ACKNOWLEDGMENTS

We thank Ane Kjeldsen and Lone Højgaard Nielsen for technical assistance and Erik Holm Nielsen, inSPIN Research Center, iNANO at Aarhus University, for assisting with the cyclization of the apelin peptides. Image acquisition and analysis were performed at the CIRB microscopy core facility. We gratefully acknowledge Jérémie Teillon at the microscopy facility.

This work was supported by the Danish Council for Independent Research, the Research School for Gene Medicine, and travel grants from the Oticon Foundation, Vestfyns Bank, and the Knud Højgaards Foundation.

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